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Horizontal and Vertical Movement of *Azospirillum brasilense* Cd in the Soil and along the Rhizosphere of Wheat and Weeds in Controlled and Field Environments

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Horizontal movement of *Azospirillum brasilense* Cd in soil and its vertical movement in the plant rhizosphere were studied. No movement was detected in the absence of living plants. In a controlled environment the bacteria moved horizontally at least 30 cm from the inoculation point to the first growing plant. Once the first root system was colonized, all the neighbouring plants became inhabited. Horizontal movement under field conditions was at least 160 cm, and depended on the presence of live plant roots. Several weeds that grew in the passes between plots acted as efficient vectors. The numbers of *A. brasilense* Cd decreased with increasing distance from the inoculated plot. Vertical movement in soil columns in a controlled environment was up to 40 cm. Under field conditions, bacteria were detected as deep as 50 cm in the root systems of wheat plants in various types of soil. During the growing season bacteria were mostly found on and in young roots at a depth of 20–50 cm and near the soil surface. A map of depth distribution of *A. brasilense* Cd showed an uneven colonization pattern within the same root system or between adjacent plants. It was concluded that *A. brasilense* Cd moved horizontally and vertically in various soil types and that this movement was mainly dependent on the presence of plants.

INTRODUCTION

The movement of beneficial rhizosphere bacteria in soil from the site of inoculation to the root system is important both for the survival of the bacteria and for successful plant inoculation. The bacteria that are applied to the soil should be motile and capable of migrating or of being moved for a certain distance and reaching the seedlings. When mature plants are inoculated the bacteria should be moved even further. Plant roots, especially those of cereals grown under semi-arid conditions, are well spread both horizontally and vertically. Since inoculation of the whole root system is impractical, the migration of the bacteria, or their effective movement, becomes a key factor that may ensure the beginning of the colonization process of the root system (Bashan, 1986*b*; Bowen & Rovira, 1976; Suslow, 1982).

Migration and/or movement of bacteria in soil has been demonstrated mainly for rhizobia inoculated onto legumes and for a few other beneficial rhizosphere bacteria. Migration distances of several centimetres, and in some cases several tens of centimetres, have been recorded (Frazier & Fred, 1922; Hamdi, 1971, 1974; Kellerman & Fawcett, 1907; Madsen & Alexander, 1982; McCoy & Hagedorn, 1979; Wong & Griffin, 1976). The migration distance of associative beneficial bacteria of the genus *Azospirillum* in natural soil has not yet been sufficiently evaluated. Bashan (1986*a*) demonstrated the migration of *Azospirillum brasilense* Cd in sterilized soil towards both wheat seedlings and synthetic attractants. Motility of *Azospirillum in vitro* towards several attractants such as sugars, amino acids, oxygen and root exudates has also been demonstrated (Barak *et al.*, 1982, 1983; Heinrich & Hess, 1985; Reinhold *et al.*, 1985).

The aim of the present study was to measure the horizontal and vertical movement of *A. brasilense* Cd under controlled and field environments in the presence or absence of wheat and weeds.

METHODS

Organisms and growth conditions. *Azospirillum brasilense* Cd (ATCC 29710) was used as a model bacterium throughout this study. Bacteria were grown as previously described (Bashan & Levanony, 1985). The peat inoculant (Nitragin, Milwaukee, USA) was produced according to Burton (1976) and contained approximately 10^8–10^9 *A. brasilense* Cd cells g^{-1}. Wheat plants (*Triticum aestivum* cv. 'Deganit') served as host in all experiments.

Experimental conditions. Plants were grown in a nethouse in flat plastic containers (80 × 15 × 15 cm) lightly filled with brown-red degrading sandy soil of Rehovot, with loess raw soil (obtained from Northern Negev) (Ravikovich, 1981) or with pure silica quartz (white sand), normally used by the glass industry (obtained from Yeruham crater, Central Negev). Six disinfected wheat seeds (treated with 1% NaOCl for 5 min, and thoroughly washed with tap water) were sown in each row (2·5 cm between seeds and 5 cm between rows). Columns made of polyethylene tubes (70 cm long, 16 cm in diameter) were filled with the same soils, except silica quartz. Each column was wrapped with aluminium foil to avoid excess heating and growth of algae, and was placed on a 320 × 50 × 50 cm stainless steel stand (16 columns per stand). A single disinfected wheat seed was sown in each column. The containers and the columns were carefully hand-irrigated at a rate equivalent to the rainfall in Rehovot during the 1982/83 season (total amount 450 mm in the winter season, according to the official meteorological data). Drip-irrigation was performed beneath a layer of vermiculite (described below), which remained dry most of the time. In short-period experiments (less than 3 weeks), the containers were irrigated only once before inoculation. These precautions were taken to avoid artifacts due to streams of irrigation water.

Bacterial inoculation. Containers were inoculated once, at the first leaf stage, with 10 ml (1×10^9 c.f.u. ml^{-1}) of double-washed *A. brasilense* Cd cells. Bashan (1986*c*) demonstrated a marked effect of bacterial inoculation on wheat seedlings when inoculated at this growth stage. The bacteria were applied into a shallow trench (2 cm depth) at the centre of each container, and then covered with soil. The whole container was then covered with a 2–3 cm layer of sterile vermiculite to prevent bacterial dispersion. The growth of plant roots towards the inoculation site was prevented by a nylon barrier (300 mesh) which did not affect movement of bacteria towards the roots. Horizontal movement beyond 20 cm was studied by applying the bacteria at one side of the container. Soil columns with no nylon barrier were identically inoculated.

Fields were inoculated immediately after seedling emergence (one to two true leaf seedlings) by dripping a suspension of bacterial peat-inoculant on the wet soil surface [0·5–2·0 g inoculant (fresh weight) m^{-2}] by means of a motor pump. A second inoculation was carried out in a similar manner 8–10 d thereafter.

Root samplings and bacterial determination. Roots of wheat and weeds were sampled by one of the following methods. Soil from either containers or columns was transversely divided into sections, 5–10 cm wide. Roots were collected from each section separately. *A. brasilense* Cd was also detected and enumerated on and in the roots of the following weeds that were found in the inoculated fields: wild oat (*Avena sterilis* L.) and wild barley (*Hordeum spontaneum* C. Koch) at Western Yizreel Valley; *Phalaris paradoxa* L. and *P. brachystachys* Lk. at Kibbutz Nir-Am (Northwestern Negev); *Malva aegyptia* L. at Kibbutz Gevulot (Western Negev); and *Notobasis syriaca* (L.) Cass. and *Silybum marianum* (L.) Gaertn., at Kibbutz Negba (Northern Negev). Roots were collected by two recently-developed tools for field sampling (Bashan & Wolowelsky, 1987): areas of 1 m^2 were sampled by the 'test-tube sampler' [three samples from the wheat plots and seven samples from the intervening pathways (5 m width)], and samples up to 50 cm deep in 5 cm intervals were provided by the 'core sampler'. Plant samples were taken during the 1983/84 season from the following soil types inoculated with *A. brasilense* Cd: alluvial soil (Negba); loess raw soil (Nir-Am) and loessial sandy soil (Gevulot). In the 1984/85 season, samples were also taken from brown alluvial soil (Moledet, eastern lower Galilee). Soil particles were removed from the roots by slight shaking and then rinsing in sterile tap water for 5 s. Bacterial enumeration was done by the ELISA technique developed specifically for *A. brasilense* Cd (Levanony *et al.*, 1987) and by the improved selection technique for *A. brasilense* in general (Bashan & Levanony, 1985). Total bacteria in the rhizosphere were determined by placing 1 g whole young roots in 100 ml Erlenmeyer flasks containing 30 ml sterile saline (0·85% NaCl). After vigorous shaking (300 strokes min^{-1}) at 30 ± 2°C for 30 min, the suspension was serially diluted and plated on solid nutrient agar supplemented with cycloheximide (200 mg l^{-1}; Difco), incubated at 25 ± 2°C for 72 h, and then counted.

Experimental design. Controlled experiments (containers and columns) were done in triplicate and were repeated two or three times. Samples of both roots and soil were tested as described above. Three plots in each field experiment (designed for evaluating the effect of various rhizosphere bacteria on wheat yield; E. Millet & M. Feldman, unpublished) were sampled. Since, in most cases, only partial root colonization took place in the field (Bashan *et al.*, 1987), the data given represent the number of bacteria calculated from root systems which had been successfully colonized by *A. brasilense* Cd. Data given are from a representative experiment. Significance is indicated by standard error.

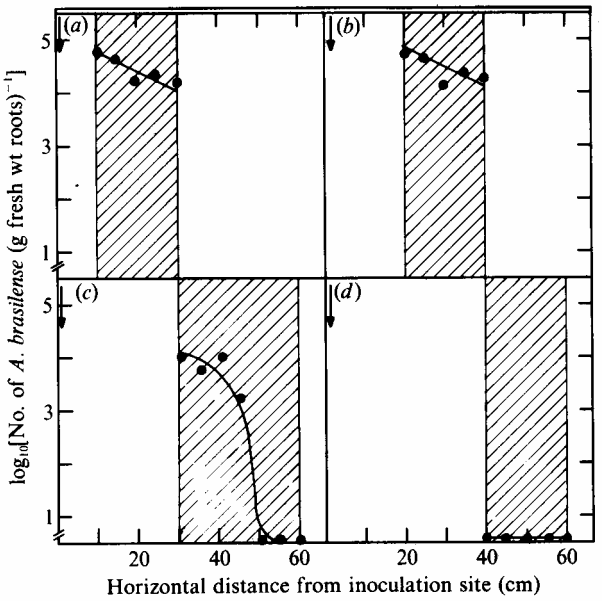

Fig. 1. Horizontal movement of *A. brasilense* Cd in containers in the direction of wheat plants at the following distances from the inoculation site: (*a*) 10 cm, (*b*) 20 cm, (*c*) 30 cm, (*d*) 40 cm. Empty areas represent areas free of plants, and hatched areas represent those with growing wheat plants. Arrows indicate inoculation sites. The number of *A. brasilense* Cd on and in roots (●) was detected by competition ELISA. Each point represents an average of six plants grown at the same distance from the inoculation site.

RESULTS

Horizontal movement of A. brasilense Cd in flat containers

Horizontal movement of *A. brasilense* Cd towards wheat plants was determined on root samples by the competition and by the indirect ELISA methods 7 d after inoculation. The maximal number of *A. brasilense* Cd on the roots detected before liquid enrichment was $6{\cdot}8 \times 10^4$ c.f.u. (g fresh wt roots)$^{-1}$. The total number of other unidentified bacteria present at the same time in the wheat rhizosphere ranged from 5×10^5 to 7×10^7 c.f.u. g (fresh wt roots)$^{-1}$. *A. brasilense* Cd moved up to 30 cm in soil free of roots, only when wheat plants were grown beyond this distance. Whenever cells of *A. brasilense* Cd reached the first line of plants in the container, all roots of the neighbouring plants became colonized (Fig. 1).

Horizontal movement of A. brasilense Cd in the field in the presence or absence of weeds

Thirty-two days after inoculation of wheat plots with *A. brasilense* Cd, samples from the plots and from the intervening pathways were obtained. The pathways were sporadically inhabited by the natural flora: *M. aegyptia* was abundant in the loessial sandy soil, whereas wild oat and wild barley were common in the colluvial–alluvial soil. *A. brasilense* Cd colonized the roots of these weeds and of four other weed species (Table 1).

A. brasilense Cd moved in loessial sandy soil and in loess raw soil from the inoculated plot through the pathways containing weeds to a distance of 160 cm; the population decreased with increasing distance from the inoculated plot. Very little bacterial movement occurred in the absence of weeds in the intervening pathways (Fig. 2*a*, *b*). No samples free of plant roots could be obtained from pathways between plots in the heavy-texture colluvial–alluvial soil. Samples from all sites along these pathways had about the same numbers of *A. brasilense* Cd (Fig. 2*c*). A similar trend was found in another heavy-texture soil (alluvial soil in Negba; data not shown).

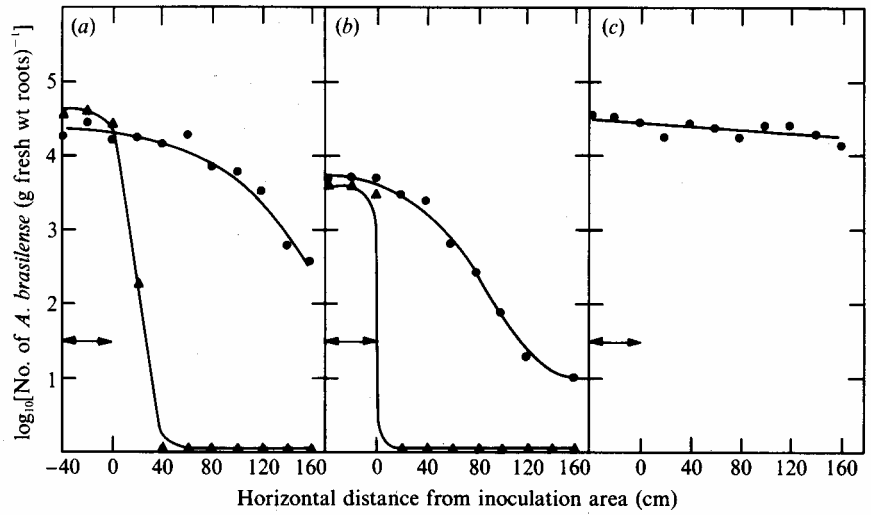

Fig. 2. Horizontal movement of *A. brasilense* Cd in (*a*) loessial sandy soil, (*b*) loess raw soil and (*c*) colluvial–alluvial soil in the field in the presence (●) or absence (▲) of weeds in the pathways between inoculated plots. Inoculated areas are indicated by arrows. Bacteria were enumerated by the improved selection technique.

Table 1. *Numbers of A. brasilense on and in roots of various weeds that grew in a wheat field, determined after inoculation of the field*

Weed species	Location	Date of sampling (in 1984)	10^{-4} × C.f.u. (g fresh wt roots)$^{-1}$†
Avena sterilis	Western Yizreel Valley	1 January	4·3 ± 2
Hordeum spontaneum			8·9 ± 0·6
Phalaris paradoxa and *Phalaris brachystachys**	Northwestern Negev	10 January	18 ± 5
Malva aegyptia	Western Negev	23 February	7·5 ± 0·8
Notobasis syriaca and *Silybum marianum**	Northern Negev	1 February	4·9 ± 0·8

* Mixed plant populations.
† Counted by the improved selection technique (Bashan & Levanony, 1985); mean number of two different samplings.

After weed foliage (*M. aegyptia*) was removed from the pathways, only very small numbers of *A. brasilense* Cd [$<10^2$ c.f.u. (g fresh wt roots)$^{-1}$] could still be found and the movement of bacteria was minimal (5 cm). The bacteria were associated with dead roots and their qualitative detection was possible only after liquid enrichment. However, even after most weed foliage was removed and only a few plants (less than four plants m^{-2}) remained, *A. brasilense* Cd moved as great a distance (160 cm) as it did in untreated pathways.

Vertical movement of A. brasilense Cd in soil columns

Columns of light-texture soil, containing or lacking plants, were sampled 8, 10, 12, 15 and 42 d after inoculation, and analysed for the presence of *A. brasilense* Cd at various depths. In the absence of plants, *A. brasilense* Cd was detected only at a depth of 2–5 cm and only less than 15 d after inoculation; at later sampling times, bacteria were undetectable even after liquid enrichment. In the presence of a growing wheat plant, *A. brasilense* Cd was detected in the plant rhizosphere as late as 42 d after inoculation, with decreasing numbers at increasing depth. At depths below 40 cm, bacteria were detected neither by the ELISA technique (Fig. 3*a*) nor by the improved selection technique. A similar trend of movement was obtained when the columns were filled with heavy loess raw soil (Fig. 3*b*), although in the non-planted columns, the bacteria survived less than 15 d.

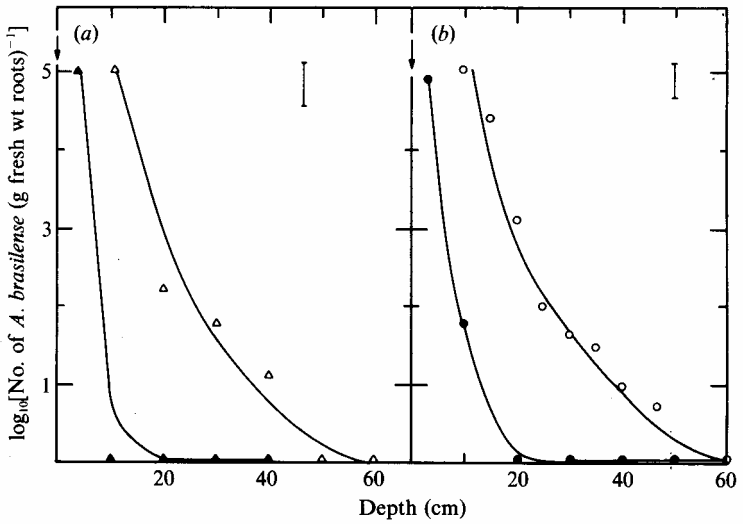

Fig. 3. Vertical movement of *A. brasilense* Cd in soil columns: (*a*) brown-red degrading sandy soil; (*b*) loess raw soil. Columns containing wheat plants (△, ○) were sampled 42 d after inoculation; columns lacking plants (▲, ●) were sampled 10 d after inoculation. The arrows indicate the bacterial application site; the bars represent the standard error of the lines. Bacteria were enumerated by the indirect-ELISA method in (*a*) and by the improved selection method in (*b*).

Vertical movement of A. brasilense Cd in several soil types

Samples of plant roots, at depths ranging from 0 to 50 cm, were obtained and analysed at various times after bacterial application. Generally, at the beginning of the season *A. brasilense* Cd was found at shallow depths (less than 20 cm). In the middle of the season (3 months after inoculation) bacteria were detected deeper (40–50 cm). No bacteria were found in soil samples free of wheat roots, even after liquid enrichment. There was variation in the distribution of bacteria within the rhizosphere of a single plant as well as between adjacent plants (Fig. 4). Only the presence of *A. brasilense* Cd is demonstrated in the depth distribution map. However, the number of *A. brasilense* Cd in roots varied between 10^3 and 10^5 c.f.u. g (fresh wt roots)$^{-1}$, compared to values of 5×10^6–6×10^7 for other rhizosphere bacteria. A similar pattern was found both in heavy- (Fig. 4*a–c*) and in light-texture (Fig. 4*d*) soils.

DISCUSSION

Horizontal and vertical movement of beneficial rhizosphere bacteria in soil can affect root colonization of the target plant. Movement of beneficial bacteria (mainly *Rhizobium*) in soil has been measured since the pioneering attempts at inoculation at the beginning of the century. Kellerman & Fawcett (1907) found that *'Bacillus radicicola'* and *'B. ochroceus'* migrated horizontally about 2·5 cm during 48 h in sterilized soils saturated with water. Frazier & Fred (1922) found that nodules of *Rhizobium* developed on soybean roots at a vertical distance of 17 cm from the inoculation site. Thornton & Gangulee (1926) demonstrated radial migration of 2·5 cm during 24 h for '*B. radicicola*' without plants; 6 d after inoculation, the horizontal migration had reached as far as 20 cm. Similarly, Brockwell *et al.* (1972) attributed the dispersal of *Rhizobium trifolii* (*R. leguminosarum* biovar *trifolii*) in plots in association with white clover to the rain water streaming over and through soil layers. Madsen & Alexander (1982) found a 10 cm vertical movement of *Rhizobium* (*Bradyrhizobium*) *japonicum* and of *Pseudomonas putida* in the presence of plants and moist soil. Smith *et al.* (1984) reported that the movement of *A. brasilense* Cd through the soil in inoculated *Sorghum* and *Pennisetum* fields was minimal, a lateral

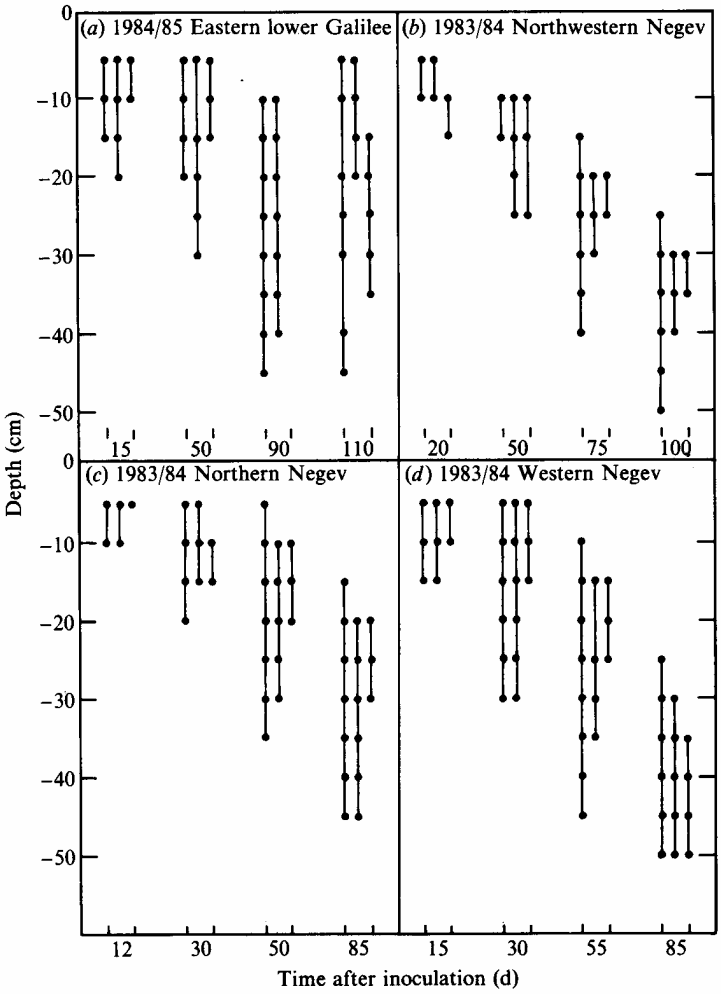

Fig. 4. Depth distribution map of *A. brasilense* Cd in wheat roots sampled at 0–50 cm depth in the field during the growing season. (*a*) Brown alluvial soil; (*b*) loess raw soil: (*c*) alluvial soil; (*d*) loessial sandy soil. Zero depth indicates the soil surface. Each vertical line represents a sampled plant; the presence (●) of *A. brasilense* Cd is shown at 5 cm depth intervals. *A. brasilense* Cd was determined by competition-ELISA in (*a*) and by the improved selection technique in (*b–d*).

movement of 15 cm being occasionally observed. Bashan (1986*a*) observed that both *A. brasilense* Cd and *Pseudomonas fluorescens* migrated through the soil towards germinating wheat seedlings to about 8 cm.

Similar patterns of movement of *A. brasilense* Cd in natural soil were detected under the controlled and field environments. No random self-motility of *A. brasilense* Cd was detected in the soil in the present study. The main factor governing horizontal and vertical movement of *A. brasilense* Cd in the soil was the presence of live plant roots, irrespective of the plant species. In the presence of roots, the bacteria moved for long distances, unlike the findings of Smith *et al.* (1984). When plant foliage had been removed and the roots were dead, horizontal transport of *A. brasilense* Cd ceased, even though the bacteria are capable of colonizing dead roots (Bashan *et al.*, 1986). Similarly, Madsen & Alexander (1982) failed to detect movement of *R. japonicum* and *P. putida* through soil in the absence of plants.

Another major factor which determines bacterial movement is the availability of a water film between soil particles. Griffin & Quail (1968) found that migration of *Pseudomonas aeruginosa* in soil was possible above field capacity and depended upon the continuity of water films. Bitton *et al.* (1974) found that water-mediated movement of *Klebsiella aerogenes* (*K. pneumoniae*) depended on the water content of the soil and on the surface properties of the bacteria. Hamdi (1974) concluded that vertical movement of *R. trifolii* was proportional to the amount of water applied to the soil. Wallace (1978) suggested that water facilitated dispersal of bacteria in soil. McCoy & Hagedorn (1979) reported that *Escherichia coli* strains moved through saturated soils due to prevailing channels. In our current field experiments, such porous structures could be formed in soil by nematodes and other microfauna usually found in wheat fields (Brown, 1984; Franklin, 1969). In addition, as a consequence of relatively high temperatures and scarcity of rain, field capacity conditions and continuous water films in the tested wheat fields were rarely found. Thus long-distance movement of bacteria in the soil towards plants would have been limited to relatively short periods, when favourable conditions prevailed. In the case of vertical movement, water limitation was less serious since the bacteria could move along the moist surfaces of the roots. Since all the experiments were done in untreated natural soil and the tested bacterial strain does not occur naturally in Israel, it is concluded that the vertical bacterial movement along plant roots took place despite competition with the native rhizosphere and soil bacteria. Thus *A. brasilense* Cd behaved as a good competitor, as was also reported earlier for *Azospirillum* strains (Balandreau, 1983; Bashan, 1986*b*).

There are some other factors that might be involved in the movement of bacteria through soil. This study does not deal directly with bacterial self-motility. Although wheat roots can excrete attractants (Ayers & Thornton, 1968), and *Azospirillum* cells are capable of both aerotaxis and chemotaxis (Barak *et al.*, 1982; Heinrich & Hess, 1985; Reinhold *et al.*, 1985), chemotaxis seems to play a more important role in bacterial movement in the soil. An oxygen gradient over long distances is less likely to be created by plants in aerated light-texture soil. Adsorption of *A. brasilense* Cd to soil particles did not prevent movement when live roots were present (unpublished data). In the current study, bacteria reached the plants after having moved through soil free of plants, despite adsorption forces acting between the bacteria and soil particles. Bashan (1986*a*) demonstrated that soil type had a marked effect on horizontal bacterial self-motility. On the other hand, under field conditions, similar patterns of vertical movement were recorded both in heavy- and in light-texture soil, implying that soil type had not affected the vertical movement of *A. brasilense* Cd along the growing roots. The effect of root growth on vertical movement of bacteria was studied by Rovira & Campbell (1974). They found that wheat tips were regularly free of bacteria, thus indicating that they did not serve as a dispersal agent. However, several studies on root colonization by *Azospirillum* showed that although most of the population was found in the elongation and root hair zones (Bashan *et al.*, 1986; Patriquin *et al.*, 1983), root tips were also colonized. Since the distance between root tips and the elongation zone is less than 1 cm, bacteria could migrate along the roots, which may act as a vector in transferring bacteria to greater depth.

Based on this study and on a previous one (Bashan, 1986*a*) it is concluded that cells of *A. brasilense* Cd can move relatively long distances both horizontally and vertically. This process was found to be dependent on the presence of plants and on the availability of water films in soil, both in controlled and in field environments. The potential of *A. brasilense* Cd to move under these favourable conditions might enhance its ability to compete with the natural rhizosphere bacteria and colonize the wheat root system.

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